Stress Hormones and Their Regulation in a Captive Dolphin Population

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LONG-TERM GOALS

This research aids our understanding of how the stress-response operates in marine mammals by evaluating markers of stress in a captive dolphin population. This research effort will determine baseline levels of putative stress hormones and evaluate the functional consequences of increased stress in the bottlenose dolphin (*Tursiops truncatus*) through the assessment of non-traditional biochemical markers.

OBJECTIVES

Marine mammals are potentially affected by multiple environmental stressors, many of which are anthropogenic. The resulting stress response is mounted to manage immediate physiological needs. When environmental stressors become chronic, however, stress-response mechanisms can become detrimental, reducing survival and reproductive effort. Assessing levels of stress and their functional consequences are therefore critical to evaluating the effects of anthropogenic disturbance on species of concern, including the influence of U.S. Naval activities on marine mammals.

This research project leverages another ONR-funded effort investigating stress markers in bottlenose dolphin at the U.S. Navy Marine Mammal Program (NMMP) and is composed of two broad components: 1) assessing baseline variability in stress hormones and 2) evaluating physiological and metabolic alterations that occur during stress in bottlenose dolphin.

The specific research objectives of this effort are to (1) establish protocols for improved sensitivity of low-level corticosteroids (aldosterone and cortisol) frequenctly observed in cetaceans; (2) determine the regulatory role of corticosteroid binding globulin (CBG) in corticosteroid action; (3) assess the role of reverse triiodothyronine (rT_3) in the counter-regulation of thyroid hormone action; and (4) determine the impact of hormone variation associated with the stress-response on the function of metabolic pathways using metabolomic analyses.

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APPROACH

Key Individuals and Collaborations

This research study is being conducted in close association with another ONR-funded effort: grant #N000141110436, *Variability of hormonal stress markers collected from a managed dolphin population*; PI: Dorian Houser, PhD; National Marine Mammal Foundation; hereafter referred to as the *Parent Project*.

Hormone assays are being conducted in collaboration with Dr Daniel Crocker at Sonoma State University; Department of Biology, Rohnert Park, CA; 94928.

Metabolomic sample processing will be conducted by *Metabolon, Inc*. and we are consulting with the Science Development Director, Jeff Buckthal (JBuckthal@metabolon.com).

Study Approach

This study capitalizes on three experimental components of the Parent Project. (1) Normal variation in stress and metabolic hormones is being evaluated by collecting samples from study dolphins throughout the year (*temporal variation*). Thirty dolphins were sampled to assess temporal variation in hormone levels. To assess the sensitivity of hormone axes, hormone stimulation experiments are underway on the (2) HPA axis, and will be performed on the (3) HPT axis (*HPA* and *HPT stimulation* studies, respectively). During these stimulation experiments, an animal's hormonal and physiological response to a simulated stressor can be evaluated. Adrenocorticotropic hormone (ACTH) is administered to stimulate the HPA axis. Similarly, the HPT axis will be activated using thyrotropin-releasing hormone (TRH) in a separate set of experiments. Subsequent sample collection will allow the investigation of both short (hours) and long-term (days) responses to HPA and HPT axis stimulation. The project described here extends the suite of biomarkers assayed in the Parent Project and attempts to improve on processing methods in order to improve quantification of certain stress biomarkers. Four project tasks are being conducted.

Task 1—Improved quantification of circulating corticosteroids (cortisol and aldosterone)
Bottlenose dolphins have low circulating levels of corticosteroids (cortisol and aldosterone). Accurate quantification requires highly sensitive assays to detect variation at or near the typical detection limit of most commercially available immunoassay kits. By modifying existing protocols, this project is evaluating assay techniques to determine the most efficient, reliable, repeatable, and cost-effective means of measuring circulating corticosteroids in bottlenose dolphin.

Task 2—Assessment of corticosteroid binding globulin
Most corticosteroids in circulation are bound with a carrier protein, primarily corticosteroid binding globulin (CBG). Only unbound hormones, however, are thought to interact with receptors and elicit a response at target tissues. Consequently, variation in carrier proteins like CBG can mediate the metabolic influence of hormones. CBG may in fact be an accurate marker of long term stress as it does not seem to vary with acute stress in some species (Chow et al, 2010). This project will therefore assess temporal variation in CBG concentration in the bottlenose dolphin.

Task 3—The influence of the HPT axis on rT_3
Variability in, and sensitivity of, the HPT axis is being investigated in the Parent Project. Under stress conditions, rT_3 production can be increased, leading to reductions in energy use by blocking T_3 receptors (Weissman, 1990). This resultant reduction in energy use may be an important energy

conserving mechanism necessary to endure stressful periods. We are therefore quantifying rT_3 concentrations for normal variation and during stimulation of the HPA and HPT axes.

Task 4—Functional analysis using metabolomics
The principal role of hormones is to influence metabolic pathways. There are numerous metabolic pathways and thousands of resultant compounds that are likely influenced by hormones associated with the stress response. Many of these compounds can be simultaneously identfied using a broad-based metabolomics technique and the identified compounds can be associated with up- and down-regulation of associated metabolic pathways (Goodacre, et al, 2004) thereby establishing some of the metabolic consequences of stress. We are therefore conducting metabolomic analyses of HPA and HPT axes stimulation to evaluate the functional consequences of increased stress in the bottlenose dolphin.

WORK COMPLETED

Funding for this study was only recently received (1 Aug 2013) and we are therefore in the early stages of this project. Sample collection for the assessment of temporal variation, from the Parent Project, is complete (735 blood collections were made in 30 study dolphins).

We have recently completed assessing various techniques to measure cortisol at low concentrations in dolphin serum and have established a simple yet reliable method to measure cortisol at concentrations less than 5 nM. We will next implement this technique to quantify cortisol concentration from samples collected in the Parent Project.

Pilot study stimulations of the HPA axis are underway in the Parent Project. We are determining an appropriate ACTH administration dose to use in bottlenose dolphins. Once this dose is established, the HPA axis stimulation experiments will begin. Samples collected from these stimulations will be used for metabolomics analysis.

HPT stimulation experiments are yet to be conducted. We anticipate those procedures early in 2014. Samples from these stimulations will be used for metabolomics analysis.
Measurement of CBG and rT_3 will be conducted in 2014 from samples previously collected in the Parent Project.

RESULTS

We have established a reliable method of measuring cortisol from dolphin serum at low circulating concentrations. This procedure modifies the protocol from a simple, affordable, and commercially available radioimmuno assay (RIA) kit (Siemens coat-a-count TKCO1). We conducted a validation of the RIA using serially diluted serum samples and found excellent parallelism with the standard curve. We determined that there were no interferring substances in dolphin samples by comparing steroid-extracted (purified) and non-extracted serum samples and found excellent agreement between the two measurements (± 9%) across a four-fold dilution. There was no detectable cortisol present in steroid-stripped serum, indicating there is no cross-reactivity with the assay antibody and other matrix compounds. We will promulgate the cortisol assay protocol in *supplementary material* with the initial peer-reviewed publication from this project to promote consistency in hormone assays within the marine mammal field.

IMPACT/APPLICATIONS

Marine mammals negatively influenced by acoustic disturbances or other U.S. Navy activities potentially experience a "stress response." The stress response can be detected by changes in stress markers, including select hormone concentrations, alterations in metabolic pathways, and, potentially, certain metabolite levels. The stress response potentially influences survival and reproduction and, therefore, may have population-level effects (Wikelski & Cooke, 2006). The additional characterization of hormones, hormone regulators, and metabolites during baseline and simulated stress conditions, as described in the current proposal, provides a mechanism by which to better detect the presence and magnitude of the physiological responses of marine mammals exposed to anthropogenic sound. In accordance with National Research Council recommendations (2005), the work described in this proposal, in concert with the Parent Project, will establish baseline and activated levels for putative stress markers in marine mammals.

RELATED PROJECTS

Grant #N000141110436, *Variability of hormonal stress markers collected from a managed dolphin population*; PI: Dorian Houser, PhD; National Marine Mammal Foundation. This project is base project from which samples for the current project are obtained.

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